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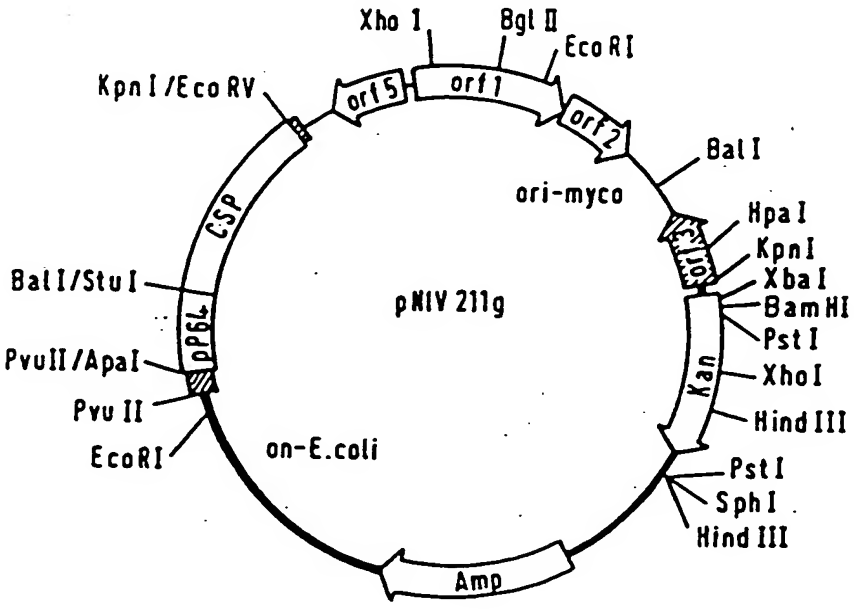

(57) Abstract

The invention provides a mycobacterial expression vector containing exogenous DNA encoding a protein of interest (e.g. an antigen) under the control of the promoter and ribosomal binding site for the 64 KD protein of *Mycobacterium bovis* BCG. Also provided are mycobacteria transformed with the mycobacterial expression vectors and vaccine compositions comprising the

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Mycobacterial expression vector

This invention relates to expression of foreign DNA in mycobacteria, to novel vectors for use in such expression and to recombinant mycobacterial vaccines.

Mycobacteria represent major pathogens of man and animals. For example, tuberculosis is generally caused in humans by Mycobacterium (M.) tuberculosis and in cattle by Mycobacterium (M.) bovis (which can be transmitted to humans and other animals, in whom it causes tuberculosis). Tuberculosis remains widespread and is an important public health problem, particularly in developing countries.

Leprosy, which is caused by M. leprae, afflicts over 10 million people, primarily in developing countries. M. tuberculosis and mycobacteria of the avium-intracellulare-scrofulaceum (MAIS) group represent major opportunistic pathogens of patients with acquired immunodeficiency disease (AIDS). M. pseudotuberculosis is a major pathogen of cattle.

On the other hand, Bacille Calmette-Guerin (BCG), an avirulent strain of M. bovis, is the most widely used human vaccine in the world and has been used as a live vaccine for more than 50 years. In the past 35 years, it has been administered to over 2.5 billion people, with remarkably few adverse effects (e.g. estimated mortality of 60/billion). BCG has been found in numerous studies to have protective efficacy against tuberculosis. Recently, however, it was found not to be effective in preventing pulmonary tuberculosis in Southern India. Mycobacterium smegmatis is a nonpathogenic bacillus which shares antigenic and adjuvent properties with BCG. Both are also reasonably easy to grow in culture.

WO 90/00594 (Whitehead Institute) describes recombinant vectors which are temperate shuttle phasmids and bacterial-mycobacterial shuttle plasmids which can be used to introduce foreign DNA stably into mycobacteria, for use inter alia as vaccine vehicles. The foreign DNA is then expressed in mycobacteria under control of the E. Coli promoters expressing kanamycin resistance, chloramphenicol resistance and cI or the M. leprae 65kD gene promoter.

It has very recently been reported that the regulatory sequences of the Mycobacterium bovis - BCG hsp 60 and hsp 70 genes can be used to drive the expression of foreign antigen genes in BCG (C.K. Stover et al, 1991).

The DNA sequence of 2431 base pairs of the Mycobacterium bovis - BCG gene encoding an immunogenic 64kD protein has been reported (Thole et al., 1987). The coding sequence begins at position 576.

It has now been found that the sequence preceding base 576 contains a promoter and ribosomal binding site which is effective in controlling expression of exogenous coding DNA in mycobacteria.

According to the present invention, there is provided a mycobacterial expression vector containing exogenous coding DNA under the control of the promoter and ribosomal binding site for the 64kD protein of Mycobacterium bovis - BCG.

The expression vector may be prepared in accordance with the invention by cleaving a mycobacterial vector to provide a linear DNA segment having a intact mycobacterial replicon, and ligating said linear segment and one or more DNA molecules which, together with said linear segment, complete

the DNA sequence for the coding DNA and the promoter and RBS for the 64kD protein of M. bovis - BCG.

In a further aspect of the invention, there is provided a recombinant mycobacterium expressing exogenous coding DNA under the control of the promoter and ribosomal binding site (RBS) for the 64kD protein of Mycobacterium bovis - BCG.

In a preferred embodiment, the 64kD promoter and RBS comprises the fragment 115-575 of the Mycobacterium bovis - BCG 64kD gene or a functional derivative thereof.

In a further aspect the invention provides the fragment 115-575 of the Mycobacterium bovis - BCG 64kD gene or a functional derivative thereof in the absence of said 64kD coding sequence and the use of said fragment or derivative for expressing exogenous coding DNA in mycobacteria.

The functional derivative may be a mutant or truncate of the 115-575 fragment which retains functional promoter and RBS activity.

It will be appreciated that it is a routine matter to probe the fragment by terminal deletions to determine the minimal structural requirement for a functional promoter and RBS.

Mutations of the 115-575 region are also contemplated in order to introduce convenient restriction sites. Thus, for example an NcoI site can be introduced at the translation start site by replacing the A at 575 by C.

The fragment may be prepared by conventional means, for example by excision of all or a part of the fragment from a vector containing the relevant part of the 64kD gene, and

ligation of excised fragments with synthetic oligonucleotide linkers as necessary to generate the required sequence, or by total oligonucleotide synthesis.

Examples of mycobacterial expression vectors of the invention include a mycobacterial plasmid or phage, or a shuttle vector comprising a linearised mycobacterial plasmid or phage fused to a linearised plasmid or phage replicable in another microorganism such as E. Coli, streptomyces, Bacillus or yeast, to aid manipulation. The principles and construction of shuttle vectors are described in W090/00594.

The recombinant mycobacterium of the invention may be prepared by transforming a mycobacterium with a mycobacterial expression vector of the invention. It will be appreciated that, depending on the nature of the expression vector of the invention, the exogenous coding DNA may be maintained extrachromosomally as a plasmid or within the mycobacterial genome via site specific integration.

For example, an E. coli cosmid may be introduced into the temperate mycobacteriophage L1, producing shuttle phasmids capable of replicating as plasmids in E. coli or lysogenizing the mycobacterial host. These temperate shuttle plasmids form turbid plaques on M. smegmatis and, upon lysogenization, confer resistance to superinfection and integrate with the mycobacterial genome.

Alternatively, a shuttle plasmid vector can be used to introduce exogenous coding DNA into mycobacteria, in which the DNA is expressed extrachromosomally. For example, M. fortuitum: E. coli hybrid plasmids may be constructed from mycobacterial and E. coli replicons containing marker genes.

Alternatively the exogenous coding DNA may be introduced into the mycobacterial genome under the control of the 64kD promoter and RBS of M. bovis - BCG but without the use of a mycobacterial expression vector, by the process of homologous recombination, site specific recombination or non-homologous recombination using a suitable vector such as the E. Coli pUC19 vector as described in W090/00594.

Mycobacteriophages useful in the present invention include L1, TM4 and D29.

It has also been found that the M. fortuitum plasmid pAL5000(Rauzier et al.) may be employed as a mycobacterial expression vector by insertion of exogenous coding DNA and a functional mycobacterial promoter and RBS in the region of ORF3-ORF4 (nucleotide bases 1541-3908).

In a further aspect of the invention there is thus provided a mycobacterial expression vector comprising pAL5000 or a replicable derivative thereof containing exogenous coding DNA and a functional mycobacterial promoter and RBS inserted in the ORF3-ORF4 region thereof, and a recombinant mycobacterium transformed with said vector.

The vector of the invention derived from pAL5000 may be prepared in accordance with the invention by cleaving pAL5000 or a derivative thereof in the region of ORF3-ORF4 and ligating the resulting linear DNA segment with one or more DNA molecules which, together with said linear segment complete the DNA sequence for the coding DNA and the promoter and RBS.

The recombinant mycobacterium may be prepared by transforming a mycobacterium with the vector of the invention derived from pAL5000.

In a preferred embodiment the functional mycobacterial promoter and RBS is the promoter and RBS for the 64kD protein of M. bovis - BCG.

A convenient site for insertion of the promoter, RBS and coding DNA is between the restriction sites EcoRV and ApaI of pAL5000.

Suitable replicable derivatives of pAL5000 include shuttle vectors in which the pAL5000 is linearised and fused to a linearised plasmid or phage replicable in another microorganism such as E. Coli to aid manipulation.

Suitable mycobacteria which may be transformed by the process of the invention include M. smegmatis, M. bovis - BCG, M. avium, M. phlei, M. fortuitum, M. lutu, M. partuberculosis, M. habana, M. scrofulaceum and M. intracellulare.

In the case of slow growing mycobacteria (e.g., M. bovis - BCG and M. tuberculosis) to be used as vaccine vehicles, it is particularly valuable to go through (i.e., introduce DNA encoding an antigen or antigens of interest into) M. smegmatis and, subsequently, into M. bovis - BCG.

There are several useful approaches to efficiently introduce the vector into a cultivable mycobacterium, such as M. bovis - BCG or M. smegmatis. For a plasmid vector, protoplast fusion can be used for efficient introduction into the mycobacterium. In this case, E. coli or streptomyces having a cloned plasmid is fused, using known techniques, with a mycobacterial spheroplast. Alternatively, E. coli minicells, which contain plasmid DNA and essentially no chromosomal DNA, can be used in carrying out a minicell protoplast fusion.

A further approach in which the vector DNA is introduced directly into intact M. smegmatis cells by electroporation obviates possible damage to mycobacterial cells which might result from use of protocols for producing sphereoplasts.

Transformation of mycobacteria by mycobacteriophage- based vectors may be carried out by phage infection procedures generally described in WO90/00594, that is, by a modification of the procedure described by Okanishi and Hopwood in relation to the preparation of spheroplasts for Streptomyces. Streptomyces, like mycobacteria, are Actinomycetales.

The modified technique is used in combination with the addition of polyethylene glycol to facilitate entry of DNA molecules into bacterial spheroplasts.

The expression vector which preferably also includes a selectable marker such as an antibiotic resistance gene is packaged into bacteriophage lambda heads using lambda in vitro packaging mix. E. coli is subsequently transduced with the phage, with the result that it is possible to screen (using antibiotic-containing medium) for colonies containing the antibiotic- resistance-encoding gene and the coding DNA.

The resulting ''library'' is introduced into M. smegmatis using, for example, electroporation. Plaques which contain vectors containing cloned insert DNA are selected. Subsequently, recombinant M. smegmatis can be used to infect a cultivable mycobacterium, such as BCG, with high efficiency. As a result, the coding DNA is introduced into mycobacterial genomic DNA, where it will be expressed.

Selection of BCG containing the coding DNA of interest can be carried out using a selectable marker, such as an antibiotic-resistance-encoding gene or a gene which

complements that missing in an auxotrophic mutant, or by use of the cI gene which encodes the repressor protein of the L1 bacteriophage. In the auxotrophy strategy, an auxotrophic mycobacterial mutant (e.g. a pyr-F mutant) is isolated and the gene present in the corresponding wild-type (nonmutant) mycobacterium is incorporated into the vector. In addition to the pyr-F mutant, it is possible to isolate deoxyglucose mutants, which have a defect in the glucokinase gene, as well as others having mutations in other biosynthetic pathways (e.g. mutations in amino acid biosynthesis, vitamin biosynthesis and carbohydrate metabolism, such as arabinose and galactose).

In either approach, a mycobacterial mutant is selected and the gene which complements the mutation is incorporated into the vector, which also contains the exogenous coding DNA.

The marker gene may be inserted in the vector under control of the same promoter as the exogenous coding DNA, i.e. to provide a polycistronic expression vector, or the marker gene may have its own promoter.

The mycobacterial mutants into which the coding DNA is successfully introduced will be identifiable (can be selected) by culturing on appropriately-selected media (e.g. media containing the antibiotic against which resistance is conferred, media containing or lacking the nutrients involved in the biosynthetic pathway affected in the mutant used) or by selecting on the basis of the appearance of plaques formed, when the cI gene is used.

It is also possible to select BCG containing coding DNA by use of monoclonal antibodies. In this case, a gene or gene fragment encoding one or more epitopes of an antigen (e.g. M. leprae or M. tuberculosis) for which monoclonal antibodies are available is introduced into the

mycobacteria. Such monoclonal antibodies are used to select for recombinant BCG containing a gene or genes encoding one or more of these epitopes. The marker antigen genes introduced in this way contain a promoter sequence and other regulatory sequences. As a result exogenous coding DNA can be added, using genetic engineering techniques, in frame, such that recombinant BCG identified by monoclonal antibodies to the marker antigen would also be expressing the exogenous coding DNA so introduced.

Another component of a plasmid vector essential in introducing exogenous coding DNA into the recombinant mycobacteria vehicle is an autonomously replicating sequence (e.g. a replicon), whose presence is a key determinant in allowing the vector to replicate autonomously (extra-chromosomally). These sequences can include, for example, a plasmid replicon or segments of a mycobacteriophage or chromosomal replication origins.

Direct introduction of exogenous coding DNA under the control of the promoter and RBS may be accomplished, for example, using minicell protoplast fusion. In this case, a selectable marker for the mycobacterium, which can be antibiotic-resistance gene or a chromosomal mutation, can be cloned into an E. coli cosmid. Also present in the E. coli cosmid will be DNA which allows efficient integration of exogenous coding DNA into mycobacterial chromosome. For example, in M. leprae, a repetitive sequence occurs which appears to be associated with recombination; analogous sequences can be identified in and isolated from BCG and M. smegmatis, incorporated into the E. coli cosmid (along with the selectable marker) and result in a high degree of recombination. Alternatively the exogenous coding DNA may be introduced directly by electroporation.

Exogenous coding DNA can be incorporated into the vector described (e.g. which includes an E. coli replicon, a segment of mycobacterial chromosomal DNA associated with recombination (a recombinogenic sequence) and two selectable markers-one serving as a marker in E. coli and the second serving as a marker in the mycobacterium). The gene(s) can then be integrated into mycobacterial chromosomal DNA, such as BCG or M. smegmatis chromosomal DNA. If the gene(s) of interest are integrated in this way into M. smegmatis, it/they can also be moved into BCG by means of a general transducing phage. In this case, it is preferable to include, in addition to the other construct components, two recombinogenic sequences: one from M. smegmatis and one from BCG.

Exogenous coding DNA is herein defined as DNA from a source other than the mycobacterium into which the DNA is being incorporated. It may code for all or a portion of a gene or genes encoding a protein(s) or polypeptide(s) of interest and/or a selectable marker or markers. The proteins or polypeptides of interest can be, for example, proteins or polypeptides against which an immune response is desired (antigen(s) of interest), enzymes, lymphokines, immunopotentiators, pharmacologic agents, steroids, and reporter molecules of interest in a diagnostic context (e.g. luciferase from a Vibrio bacterium or of firefly origin; β-galactosidase; β-glucoronidase; catechol dehydrogenase). The recombinant mycobacteria of the invention are particularly useful as vehicles by which the DNA of interest can be expressed. Such vehicles can be used, for example, as vaccine vehicles which express a polypeptide or a protein of interest (or more than one polypeptide or protein), such as an antigen or antigens, of one or more pathogens of interest. The recombinant mycobacteria can also be used as a vehicle for expression of immunopotentiators, enzymes, pharmacologic agents, steroids and antitumor agents; for expression of a polypeptide or a protein useful in producing

stress proteins, which can be administered to evoke an immune response or to induce tolerance in an autoimmune disease (e.g. rheumatoid arthritis). Recombinant mycobacteria can, for example, express protein(s) or polypeptide(s) which are growth inhibitors or are cytocidal for tumor cells (e.g., interferon α, β or γ; interleukins 1-7, tumor necrosis factor (TNF) α or β and, thus, provide the basis for a new strategy for treating certain human cancers (e.g., bladder cancer, melanomas). Pathogens of interest include any virus, microorganism, or other organism or substance (e.g., a toxin or toxoid) which causes disease.

The present invention thus relates to a process for preparing a protein or polypeptide of interest which process comprises expressing exogenous DNA in a recombinant mycobacterium according to the invention and, if required, recovering the protein or polypeptide of interest, and to protein or polypeptide of interest produced in a recombinant mycobacterium according to the invention. For the production and recovery of protein or polypeptides of interest, M. smegmatis is a preferred mycobacterium.

The present invention also relates to methods of vaccinating a host with the recombinant mycobacterium to elicit protective immunity in the host. The recombinant vaccine can be used to produce humoral antibody immunity, cellular immunity (including helper and cytotoxic immunity) and/or mucosal or secretory immunity. In addition, the present invention relates to use of the antigens expressed by the recombinant cultivable mycobacterium as vaccines or as diagnostic reagents.

Mycobacteria have adjuvant properties among the best currently known and, thus, stimulate a recipient's immune system to respond to other antigens with great effectiveness. This is a particularly valuable aspect of

the vaccine because it is expected to induce cell- mediated immunity and, thus, has the potential to provide immunity against pathogens in cases where cell- mediated immunity appears to be critical for resistance. Second, the vaccine is expected to stimulate long-term memory or immunity. As a result, a single (one-time) inoculation has the potential to produce long-term sensitization to protein antigens. The vaccine vehicle of the present invention has the potential to prime long-lasting T cell memory, which stimulates secondary antibody respones neutralizing to the infectious agent or the toxin. This is useful, for example, against tetanus and diphtheria toxins, pertussis, malaria, influenza, herpes viruses, snake and insect venoms, leprosy, tuberculosis, diphtheria, tetanus, leishmania, salmonella, schistomiasis, measles, mumps, respiratory syncitial virus, varicella zoster virus, treponema, shigella, neisseria, borellia, rabies, polio, HIV and vibrio cholerae. Genes encoding one or more protective antigens for one or more of the disease-causing pathogens can be obtained by isolation of the naturally-occurring DNA (e.g. from the pathogenic organism or toxin-producing organism); by cloning and amplification of the DNA sequence of interest, using known genetic engineering techniques (such as polymerase chain reaction or see, for example, Maniatis, T. et al Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y. (1982).); or by mechanical synthesis, and introduced into the mycobacterium.

A particular antigen of interest is the Plasmodium CS protein gene (US 4707357, Airnot et al.) more particularly the P. falciparum CS protein gene. In one embodiment the antigen is expressed as ala_{18}-ser_{391} with an N-terminal methionine. In another embodiment the antigen is expressed as the full length protein met_1-asn_{412}.

In further aspects the invention provides a Plasmodium CS protein gene expression unit comprising a DNA coding sequence for said protein and a regulatory element necessary for transcription of the coding sequence and translation within a mycobacterium, a mycobacterial vector comprising said gene expression unit and a recombinant mycobacterium comprising said gene expression unit.

In a preferred aspect the vector contains the Plasmodium CS protein gene under the control of the promoter and RBS for the 64kD protein of M. bovis - BCG. In another preferred aspect the gene is carried within pAL5000 or a replicable derivative thereof containing a functional mycobacterial promoter and RBS in the ORF3-ORF4 region thereof..

In one aspect, the recombinant mycobacterium preferably comprises the Plasmodium CS protein gene under the control of the promoter and RBS for the 64kD protein of M. bovis - BCG.

In another aspect the recombinant mycobacterium is preferably transformed with pAL5000 or a replicable derivative thereof containing a mycobacterial promoter and RBS and said gene in the ORF3-ORF4 region thereof.

It will, however, be appreciated that expression of the CS protein gene expression unit may also be achieved by procedures generally described in WO90/00594.

In any of the uses of the recombinant mycobacteria to express a protein or polypeptide, it is possible to include in the shuttle vector DNA encoding a signal sequence and thus, provide a means by which the expressed protein or polypeptide is made in the cytoplasm and then secreted in the cell walls. For example, the signal sequence from α

antigen, which is secreted in mycobacteria, could be used. Alternatively, the signal sequence for β-galactosidase, agarase or α amylase could be used.

BCG has important advantages as a vaccine vehicle in that: 1) it is the only childhood vaccine currently given at birth; 2) in the past 40 years, it has had a very low incidence of adverse effects, when given as a vaccine against tuberculosis; and 3) it can be used repeatedly in an individual (e.g. in multiple forms).

A further advantage of BCG in particular, as well as mycobacteria in general, is the large size of its genome (approximately 3 x 10^6 bp in length). Because the genome is large, it is able to accommodate a large amount of exogenous DNA and, thus can be used to make a multi-vaccine vehicle (i.e. one carrying DNA encoding protective antigens for more than one pathogen or toxin.)

Accordingly, a preferred mycobacterial host cell for use as a vaccine vehicle is M. bovis - BCG.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al, University Park Press, Baltimore, Maryland, U.S.A., 1978. Encapsulation within liposomes is described, for example by Fullerton, U.S. Patent 4,235,877.

The amount of the recombinant mycobacteria present in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccines. Such amount will vary depending upon which specific immunogen is employed. Generally, it is expected that each dose will comprise 1-1000 µg of protein, preferably 1-200 µg. An optimal amount for a particular vaccine can be ascertained by

standard studies involving observation of antibody titres and other responses in subjects. Following an initial vaccination, subjects will preferably receive a boost in about 4 weeks, followed by repeated boosts every six months for as long as a risk of infection exists.

The invention also provides a vaccine composition which comprises recombinant mycobacteria according to the invention in combination with a pharmaceutically acceptable carrier, recombinant mycobacteria according to the invention for use in vaccinating a host and use of recombinant mycobacteria according to the invention in the preparation of a vaccine.

The invention will now be illustrated by reference to the following examples.

Example 1

Plasmid pNIV2119 expressing circumsporozoite protein of P. falciparum (amino acids 18 to 391)

1.1 Preparation of starting materials

(a) Plasmid pAL8

pAL8 (Fig. 1) is a shuttle vector constructed by B. Gicquel at the Institut Pasteur of Paris. (Unité de Génie Microbiologique, 28 rue du Dr. Roux, F-75724 Paris, Cedex 15). It consists of the M. fortuitum pAL5000 plasmid in the KpnI unique restriction site of which (position 1983 of pAL5000 sequence published by Rauzier et al) plasmid pTZ19R (an E. Coli plasmid containing an origin of replication and ampicillin resistance gene purchased from Pharmacia) linearised through its unique KpnI site was inserted. Moreover, the Kan^r resistance gene (from Tn903) was isolated from pUC4K (purchased from Pharmacia) as a PstI fragment and introduced into the pTZ19R unique PstI site. pAL8 has 8947 bp.

(b) Plasmid pNIV2116

(i) A blunted MluI-BamHI fragment from the plasmid pRIB1000 (Thole et al., 1985 and Thole et al., 1987) was subcloned into pUC19 (Yanisch-Perron et al.) cut with HindII, to yield an intermediate construct named pRIB-pUC. The MluI-BamHI fragment contains the 5' untranslated region of the P64 gene of pRIB1000 up to the coding sequence.

pRIB-pUC. This fragment starts at position 115 and terminates at 579 with the ATG and the first base (G) of the second codon of P64.

(ii) An StuI-BclI fragment of 1120 bp was excised from pNIV2104 (WO 90/07006) coding for the circumsporozoite protein of Plasmodium falciparum (US 4707357). This fragment starts at the second base of the triplet coding for ala_{18} of the circumsporozoite protein and stops after ser_{391}:

391 stop BclI
AGT TGA TGATCA

(iii) Fragments from (i) and (ii) were ligated to produce an expression cassette which was introduced between the AvaI and BamHI sites of plasmid pULB1221 (EP0186643 Region Wallonne) to give plasmid pNIV2116.

1.2 Preparation of plasmid pNIV2119 and pNIV2126

The expression cassette was isolated from the plasmid pNIV2116 from 1.1(b) by restriction with KpnI and PvuII. This fragment, bases 115-579 of P64 preceded by five bases (CTGAG) from the polylinker of pULB1221 and followed by the coding sequence for the circumsporozoite protein gene starting at the second base of the ala_{18} coding triplet, was introduced into pAL8 from 1.1 (a) between EcoRV and ApaI unique restriction sites at positions 6145 and 7986 respectively (corresponding to positions 2035 and 3876 of pAL5000) resulting in the replacement of a 1841 bp non-essential fragment of the plasmid by the expression cassette to give plasmid pNIV2119 (Fig. 2) and plasmid pNIV2126 (containing the expression cassette in the opposite orientation).

1.3 Expression of circumsporozoite protein in M. smegmatis

Plasmids pNIV2119 and pNIV2126 from 1.2 were introduced into M. smegmatis by electroporation. Transformants were selected on the basis of their resitance to kanamycin. They were shown by ELISA and Western blot (Chan, D.W.; Towbinet et al.) to express the circumsporozoite protein of P. falciparum at a level of about 0.6% of total cell proteins, whatever the orientation of the expression cassette.

1.4 Expression of circumsporozoite protein in BCG

Plasmid pNIV2119 from 1.2 was introduced into BCG by electroporation. Transformants were selected on the basis of their resistance to kanamycin and by hybridization with a CSP probe. They were shown by ELISA and Western blot to express the circumsporozoite protein of P. falciparum at a level of about 0.15% of total cell proteins.

Example 2

Plasmids pNIV2124 and pNIV2125 expressing the complete circumsporozoite protein of P. falciparum

2.1 Preparation of plasmids pNIV2124 and pNIV2125

(i) A XhoI -Asp700 fragment of 450 bp was excised from pRIB-pUC (Example 1.1(b)(i)). This fragment starts at position 115 and terminates at 567, 8 bases upstream from the ATG. It was ligated to a synthetic fragment of the following sequences (90009/90010).

```
                Met Met Arg Lys Leu Ala Ile Leu Ser Val
90009 ACTTCGCA ATG ATG AGA AAA TTA GCT ATT TTA TCT GTT
90010 TGAAGCGT TAG TAC TCT TTT AAT CGA TAA AAT AGA CAA
                                    XhoI protruding end

Ser Ser Phe Leu Phe Val Glu Ala
TCT TCC TTT TTA TTT GTT GAG GCC T
AGA AGG AAA AAT AAA CAA CTC CGG A AGCT
                          StuI
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and inserted into the XhoI site of pNIV103, a derivative of pULB1221 in which the replicon from pBR322 was replaced by that of pUC19, leading to pNIV2223.

The synthetic fragment codes for aa 1 to 18 of CSP preceded by the 8 last bases of the P64 promoter. The Stu. site overlapping aa17 and aa18 is immediately followed by an XhoI protruding end.

(ii) A StuI-DdeI fragment was isolated from pNIV2107 (WO 90/07006). This fragment starts at the second base of the triplet coding for Ala18 and stops 17 bases downstream to the natural stop (TAG) codon of CSP. After blunting of its DdeI extremity, this fragment was inserted into the StuI site of pNIV2223 leading to pNIV2123 (Fig. 3a). This plasmid thus contains the expression cassette consisting of the p64 promoter and Shine-Dalgarno sequences (positions 115 to 575)
followed by the sequence coding for the complete CSP.

(iii) The expression cassette was excised from pNIV2123 as a PvuII-MscI fragment and was inserted into pAL8 between the blunted ApaI and EcoRV sites resulting into plasmids

pNIV2124 and pNIV2125 (the expression cassette being in the opposite orientation) (Fig. 3b).

2.2 Expression of the complete CSP in M. smegmatis

Plasmid pNIV2124 was electroporated into M. smegmatis cells and recombinant colonies were selected on the basis of their resistance to kanamycin. They were shown by ELISA and Western blot to express the CSP at about 0.3% of total cell proteins.

2.3 Expression of the complete CSP in BCG

Plasmid pNIV2124 was electroporated into BCG and recombinant colonies were selected on the basis of their resistance to kanamycin and by hybridization with a CSP probe. They were shown to express the complete circumsporozoite protein of P. falciparum at about 0.01% of total cell proteins..

Example 3

Plasmids pNIV2120 and pNIV2121 expressing circumsporozoite protein of P. falciparum

3.1 Preparation of plasmids pNIV2120 and pNIV2121

The construction of plasmids pNIV2120 and pNIV2121 was similar to that of pNIV2119 in Example 1 except that the fragment of the 5' untranslated region of the P64 gene excised from pRIB-pUC was the XhoI-Asp700 fragment which terminates at position 567. This fragment is followed by synthetic oligonucleotides to restore the complete sequence up to the ATGG (position 579). The only difference in the resulting sequence with respect to the natural one is the

presence of a C instead of an A immediately before the A of the ATG at position 575, thus introducing a convenient NcoI restriction site. The sequence of the synthetic fragment is as follows:

5'ACTTCGCCATGG 3'
3'TGAAGCCGTACC 5'

The XhoI-Asp700 fragment and the synthetic fragment were first inserted between the XhoI and MscI sites of pNIV103.

The resulting plasmid pNIV2221 (Fig. 4) was tested for the reconstitution of Asp700, the presence of NcoI and the reconstitution of MscI. The StuI-BclI fragment coding for the circumsporozoite protein was inserted in plasmid pNIV2221 between MscI and BamHI. The complete expression cassette was then transferred into pAL8 as a PvuII-Asp718 fragment (coding for the CSP molecule starting at the Ala18 residue) between ApaI and EcoRV as described in 1.2 above. Plasmids were obtained with the expression cassette in both orientations (pNIV2120 (Fig. 5) and pNIV2121).

3.2 Expression of circumsporozoite protein in M. smegmatis

Plasmid pNIV2120 was electroporated into M. smegmatis and shown to direct the expression of the CSP at the same level as that obtained with pNIV2119 and pNIV2125.

3.3 Expression of circumsporozoite protein in BCG

Plasmid pNIV2120 was electroporated into BCG. Transformants were selected on the basis of their resistance to kanamycin. They were shown by ELISA and Western blot to express CSP at

the same level as that obtained with pNIV2119 and pNIV2125.

Example 4

Expression of the complete circumsporozoite protein of P.falciparum from integrated plasmid pNIV2141

4.1 Construction of plasmid pNIV2141

a) Plasmid pNIV2203 is a pUC19 based plasmid containing a 2320 bp genomic fragment of BCG DNA containing the gene coding for Ornithine Carbamoyl transferase. This plasmid was constructed by subcloning of a blunted NdeI-BglII fragment from pOTC into the HindII site of pUC19. pOTC itself was selected by complementation of an OTC minus E. coli strain described in Crabeel et al (Gene 5, 207-231, 1979) by the DNA of a genomic library constructed by insertion of Sau3A partially digested BCG DNA into the BamHI site of pASI.

b) pNIV2141 (see Fig 6) was constructed by ligation of the Kan^R cassette isolated as a HindII fragment from pUC4K (Pharmacia) and the P64-$CSPs^+a^+$ cassette isolated as a PvuII-MscI fragment from PNIV2123 into a unique blunted XhoI restriction site into the OTCase coding sequence of pNIV2203.

4.2 Expression of circumsporozoite protein in BCG

Plasmid pNIV2141 was introduced into BCG by electroporation. Transformants were selected on the basis of their resistance to kanamycin and by hybridization with a CSP probe. They were shown by ELISA and Western blot to express the circumsporozoite protein of P.falciparum. Plasmid pNIV2141

was shown by Southern blot analysis to be integrated into the BCG genome by non-homologous recombination.

.....

In the figures:

Figure 1

Plasmid pAL8

———	vector sequences from pAL5000
▬▬	vector sequences from pTZ19R
▨	pAL5000 ORF3
▨	pAL5000 ORF4
Kan^r	kanamycin resistance gene
Amp^r	ampicillin resistance gene

Figure 2

Plasmid pNIV2119

pP64 - nucleotides 115-579 of M. bovis - BCG 64kD gene preceded by five bases from the polylinker of pULB1221

CSP - coding sequence for the P. falciparum circumsporozoite protein starting at the second base of the ala_{18} coding triplet and ending at ser_{391} followed by the TAG stop codon followed by TGATCCATG from the polylinker of pNIV103 (PvuII)

Other features as described in Figure 1.

Figures 3a and b

Preparation of Plasmids pNIV2123, 2124 and 2125

P64 - nucleotides 115-567 of M. bovis - BCG 64kD gene

oligo(nucleotides) - synthetic oligonucleotides 90009/90010

CSP - coding sequence for the P. falciparum circumsporozoite protein starting at the second base of the ala_{18} coding-triplet and ending 17 bases downstream to the natural stop codon (TAG) of the protein.

$CSPs^{+}a^{+}$ - coding sequence for the complete (amino acids 1 to 412) circumsporozile protein of P.falciparum.

Figure 4

Plasmid pNIV2221

ORI - replicon from pUC19

P64 - nucleotides 115-567 of M. bovis - BCG 64kD gene

Synthetic fragment - 5'ACTTCGCCATGG 3'
3'TGAAGCCGTACC 5'

Figure 5

Plasmid pNIV2120

P64 - nucleotides 115-579 of M. bovis - BCG 64kD gene preceded by five bases from the polylinker of pULB1221 and with a C at position 575

CSP - coding sequence for the P. falciparum circumsporozoite protein starting at the second base of the ala_{18} coding triplet and ending at ser 391 followed two TGA stop codons and the sequence TCCATG originating from the polylinker of pNIV103.

Figure 6

Preparation of plasmid pNIV2141

P64 - nucleotides 115-575 of M. bovis - BCG 64 kD gene preceded by five bases from the polylinker of pULB1221.

$CSPs^+a^+$ - coding sequence for the complete (amino acids 1 to 412) circumsporozoite protein of P.falciparum.

OTC - genomic BCG fragment containing the OTCase gene.

References

Airnot et al., Science 1985 230 815

Chan, D.W., Immunoassay, A Practical Guide, Academic Press Inc. 1987.

Collins, P.L. et al., J. Virol. 1985 54(1)65

Davison, A.J. et al., J. gen. Virol. 1986 67 1759

Felger, J.M. et al., J. Virol. 1988 62(6)2076

Openshaw, P.J.M. et al., J. Virol. 1990 64(4)1683

Rauzier, J. et al., Gene 1988 71 315

Stover, C.K. et al., Nature, 1991, 351, 456 - 460

Thole, J.E.R. et al. (1985), Infection and Immunity 1985 50(3)800

Thole, J.E.R. et al. (1987), Infection and Immunity 1987 55(6)1466

Towbin, H. et al., Proc. Natl. Acad. Sci. USA 1979 76 4350

Yanisch-Perron, C. et al., Gene 1985 33 103

Claims

1. Recombinant DNA comprising DNA exogenous to the genome of a mycobacterium, the said DNA encoding a protein and being under the control of the promoter and ribosomal binding site of the 64 KD protein of M. bovis - BCG.

2. A recombinant vector comprising DNA as claimed in Claim 1.

3. A vector as claimed in Claim 2 which is suitable for introduction of exogenous DNA into the genome of a mycobacterium.

4. A vector as claimed in claim 2 or Claim 3 which is a mycobacterial expression vector.

5. A vector as claimed in Claim 4 comprising a mycobacterial plasmid or mycobacteriophage.

6. A vector as claimed in Claim 5 comprising a shuttle vector in which the mycobacterial plasmid or mycobacteriophage is linearised and fused to a linearised plasmid or phage replicable in a micro-organism other than mycobacterium.

7. A vector as claimed in any one of claims 4 to 6 in which the promoter and ribosomal binding site comprise the fragment 115 to 575 of the Mycobacterium bovis - BCG 64 KD gene or a functional derivative thereof.

8. A vector as claimed in Claim 7 in which the said functional derivative is a mutant or truncate of the 115 to 575 fragment which retains functional promoter and ribosomal binding site activity.

9. A vector as claimed in any one of claims 4 to 8 comprising the M. fortuitum plasmid pAL 5000 or a replicable derivative thereof into which the exogenous coding DNA and the promoter and ribosomal binding site have been inserted in the region of ORF3 - ORF4 (bases 1541 - 3908).

10. A vector as claimed in any one of Claims 2 to 9 in which the exogenous DNA encodes an antigen.

11. A vector as claimed in Claim 10 in which the antigen is Plasmodium circumsporozoite protein.

12. A recombinant mycobacterium transformed with a vector as claimed in any one of claims 2 to 11.

13. A recombinant mycobacterium as claimed in Claim 12 selected from the group consisting of M. smegmatis, M. bovis - BCG, M. avium, M. phlei, M. fortuitum, M. lufu, M. partuberculosis, M. habana, M. scrofulaceum and M. intracellulare.

14. A recombinant mycobacterium as claimed in Claim 13 which is M. bovis - BCG.

15. A process for preparing a recombinant mycobacterium which process comprises transforming, under suitable conditions, the mycobacterium with a recombinant vector as claimed in any one of Claims 2 to 11.

16. A process for preparing a protein encoded by DNA exogenous to the genome of a mycobacterium, which process comprises expressing the exogenous DNA in a recombinant mycobacterium as claimed in any one of Claims 12 to 14.

17. A process as claimed in Claim 16 in which the recombinant mycobacterium is M. smegmatis.

18. A vaccine composition comprising a recombinant mycobacterium as claimed in any one of claims 12 to 14 together with a pharmaceutically acceptable carrier.

19. A recombinant mycobacterium as claimed in any one of claims 12 to 14 for use in vaccinating a human or animal.

20. A method of eliciting protective immunity in a host, which method comprises vaccinating the host with an effective amount of a vaccine composition as claimed in claim 18.

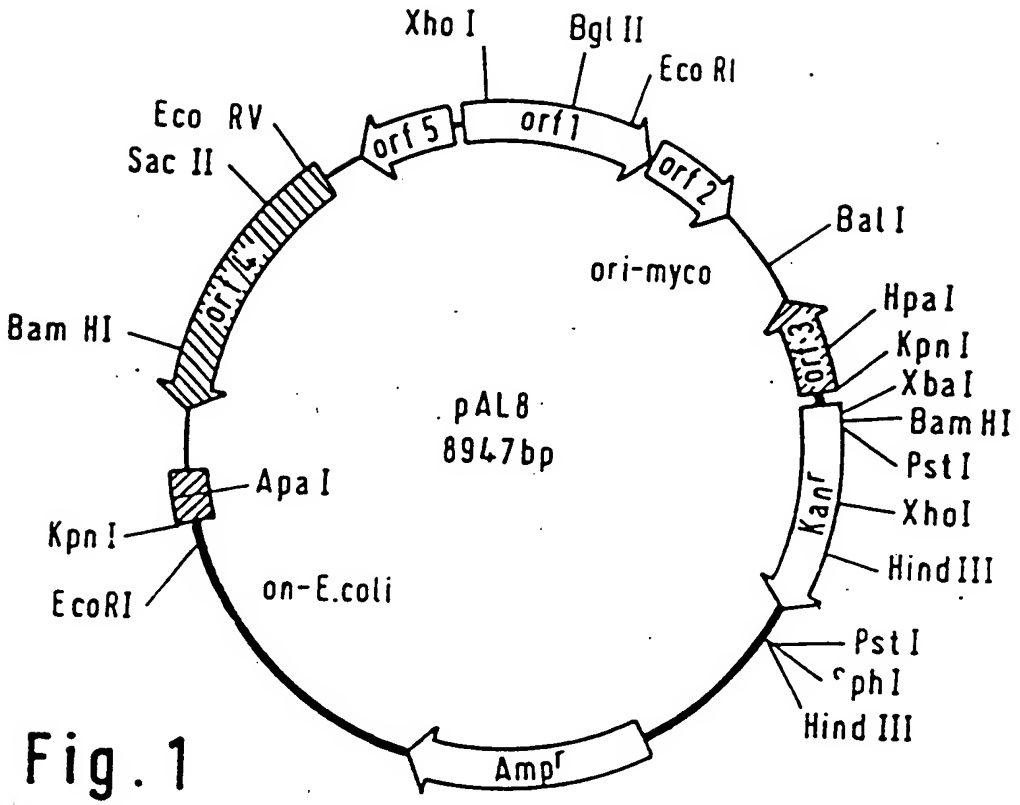

Xho I
Bgl II
Eco RI
Eco RV
Sac II
orf 5
orf 1
orf 2
Bal I
ori-myco
Hpa I
Kpn I
Xba I
Bam HI
Pst I
Xho I
Hind III
Pst I
Sph I
Hind III
Amp r
Kan r
orf 3
orf 4
Bam HI
Apa I
Kpn I
EcoRI
on-E.coli
pAL8
8947bp

Fig. 1

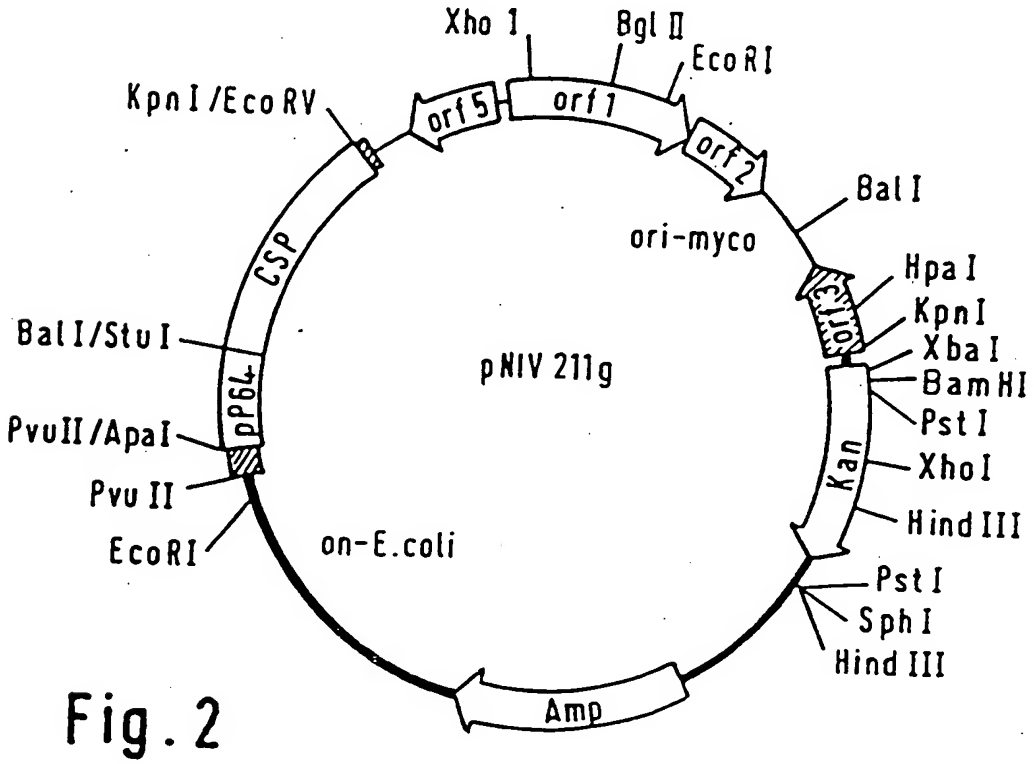
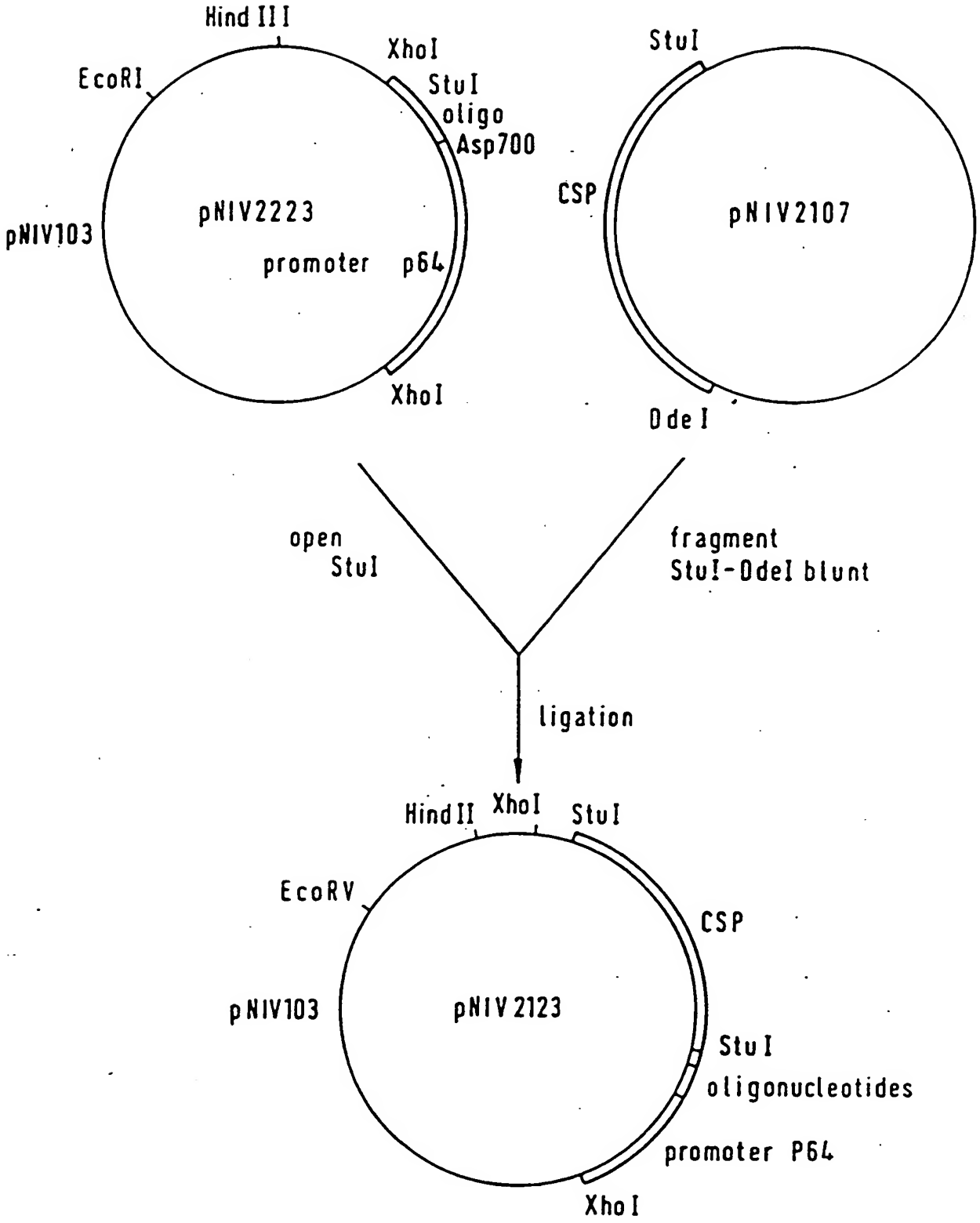

Xho 1
Bgl II
EcoRI
KpnI/EcoRV
orf5
orf1
orf2
Bal I
ori-myco
Hpa I
KpnI
Xba I
BamHI
Pst I
Xho I
Hind III
Pst I
Sph I
Hind III
Amp
Kan
orf3
CSP
pP64
BalI/StuI
PvuII/ApaI
Pvu II
EcoRI
on-E.coli
pNIV 211g

Fig. 2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XhoI
EcoRI
StuI
oligo
Asp700
pNIV103
pNIV2223
promoter
p64
XhoI
StuI
CSP
pNIV2107
OdeI
open
StuI
fragment
StuI-OdeI blunt
ligation
HindII
XhoI
StuI
EcoRV
CSP
pNIV103
pNIV2123
StuI
oligonucleotides
promoter P64
XhoI

Fig. 3a

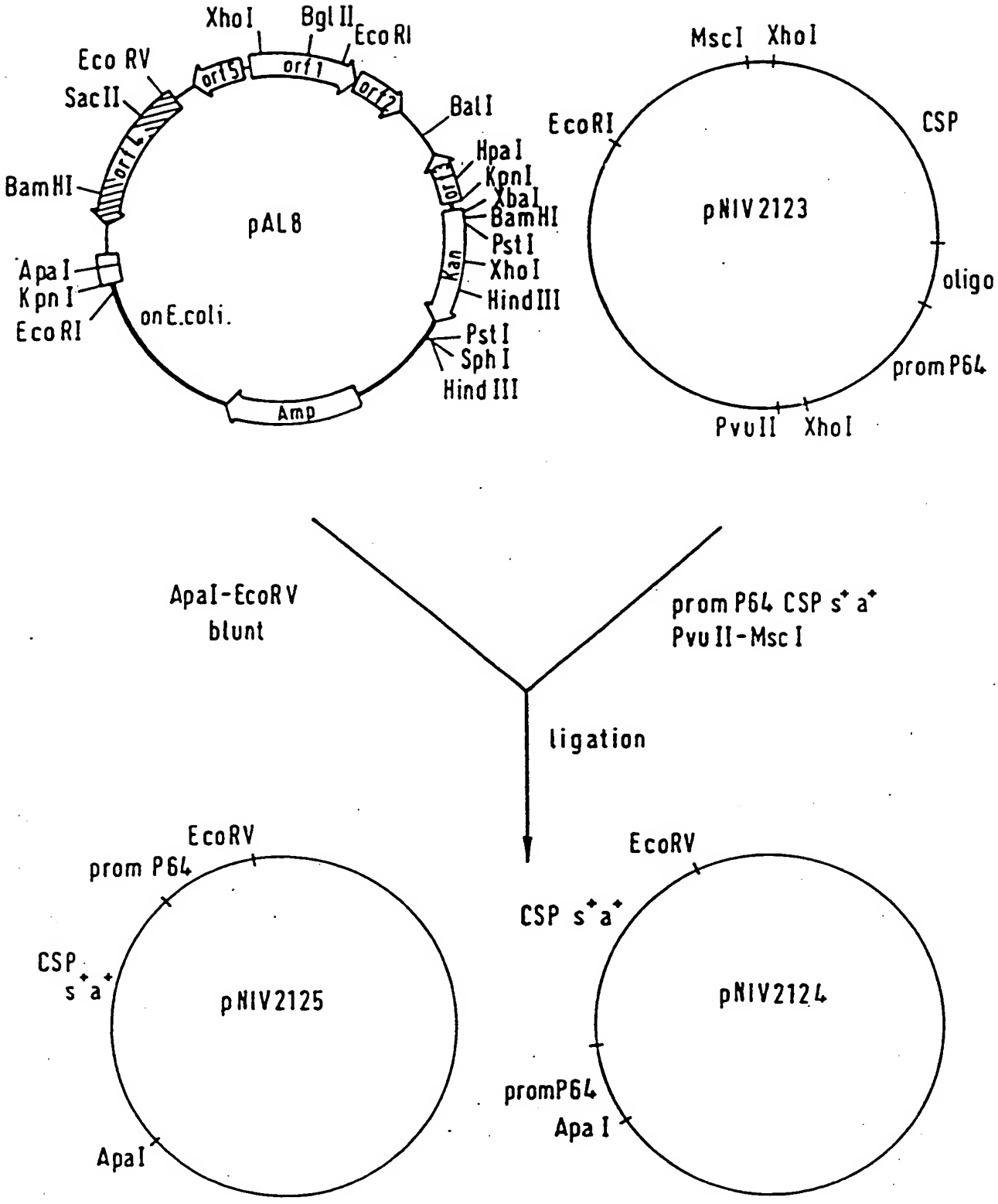
XhoI
BglII
EcoRI
Eco RV
SacII
orf5
orf1
orf2
BalI
HpaI
KpnI
XbaI
BamHI
PstI
XhoI
HindIII
PstI
SphI
HindIII
BamHI
orf6
pAL8
ApaI
KpnI
EcoRI
onE.coli.
Kan
Amp
MscI
XhoI
EcoRI
CSP
pNIV2123
oligo
promP64
PvuII
XhoI
ApaI-EcoRV
blunt
promP64 CSP s+a+
PvuII-MscI
ligation
EcoRV
promP64
CSP
s+a+
pNIV2125
ApaI
EcoRV
CSP s+a+
pNIV2124
promP64
ApaI

Fig. 3b

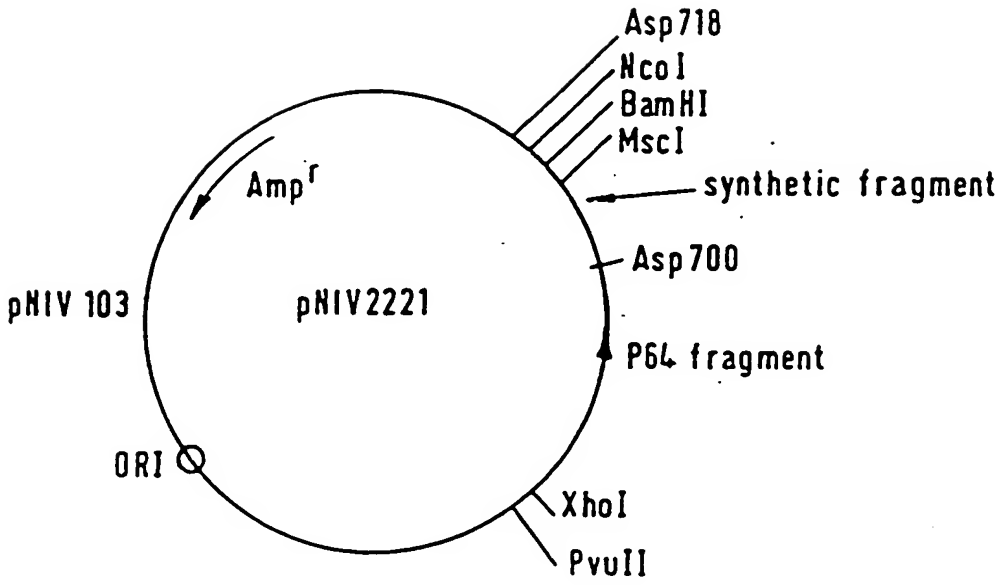

Fig. 4 Structure of pNIV2221

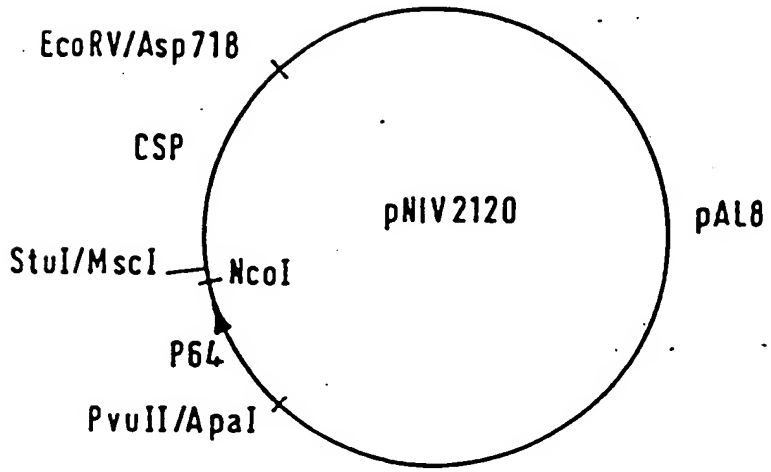

Fig. 5 Structure of pNIV2120

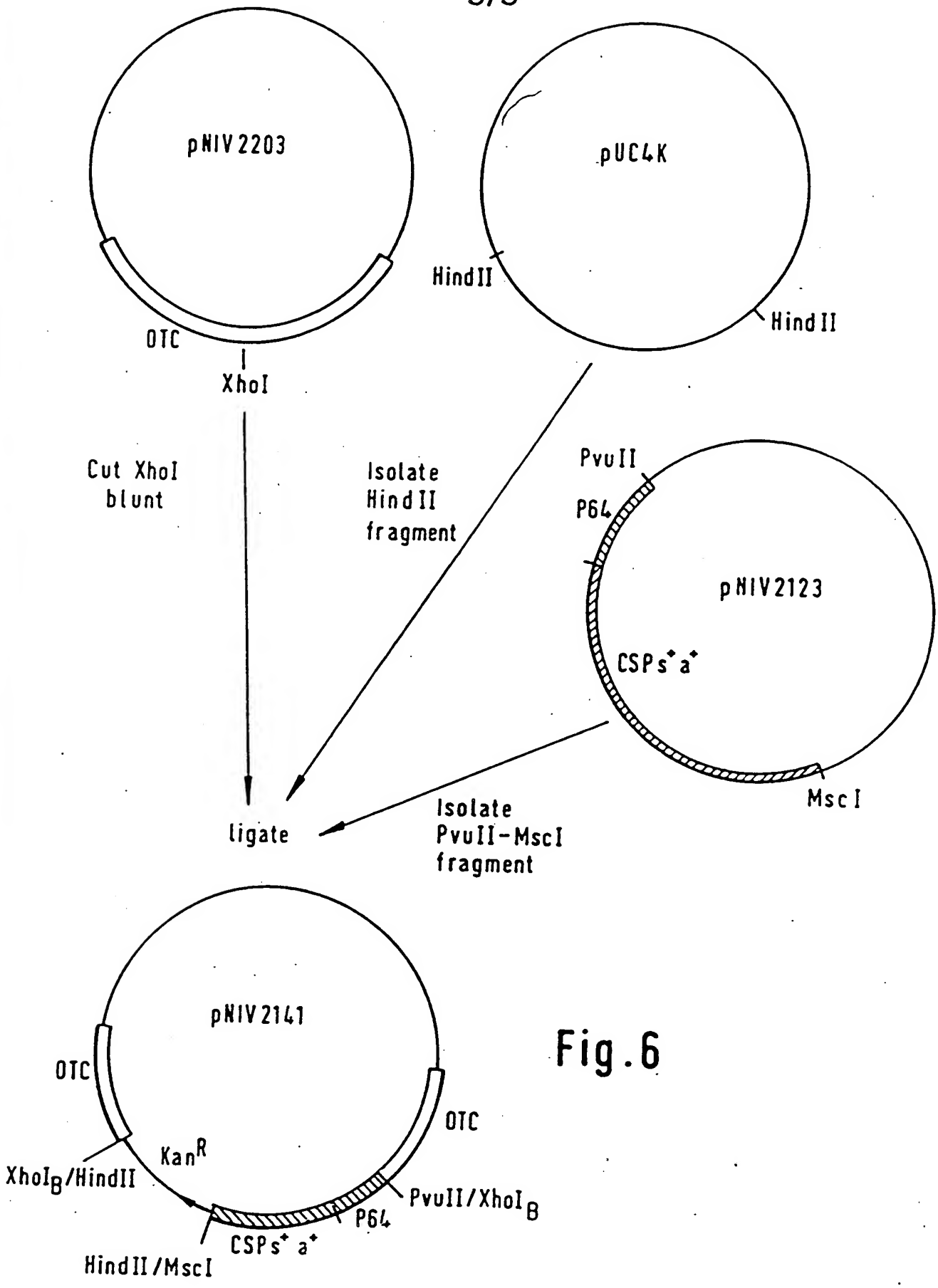
pNIV2203
OTC
XhoI
pUC4K
HindII
HindII
Cut XhoI
blunt
Isolate
HindII
fragment
PvuII
P64
pNIV2123
CSPs⁺a⁺
MscI
ligate
Isolate
PvuII-MscI
fragment
pNIV2141
OTC
OTC
Kan^R
XhoI_B/HindII
PvuII/XhoI_B
P64
CSPs⁺ a⁺
HindII/MscI

Fig.6

INTERNATIONAL SEARCH REPORT

International Applica No PCT/EP 91/01332

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)*

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 N 15/74 C 12 N 15/30 A 61 K 39/04
C 12 P 21/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	Proc. Natl. Acad. Sci. USA, volume 85, September 1988, (Washington, US), S.B. Snapper et al.: "Lysogeny and transformation in mycobacteria: stable expression of foreign genes", pages 6987-6991, see abstract; page 6988, left-hand column, line 59 - right-hand column, line 8 ---	1-19
Y	Infection and Immunity, volume 55, no. 6, June 1987, (Washington, US), J.E.R. Thole et al.: "Characterization, sequence determination, and immunogenicity of a 64-kilodalton protein of mycobacterium bovis BCG expressed in Escherichia coli K-12", pages 1466-1475, see page 1472, left-hand column, lines 13-34; page 1472, left-hand column, lines 48-65 (cited in the application --- -/-	1-19

* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
07-09-1991	20. 11. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	WO,A,9000594 (WITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 25 January 1990, see abstract; page 75, line 22 - page 76, line 5; claims 1,6,9 ---	10-11, 18-19
A	EP,A,0166410 (THE UNITED STATES OF AMERICA) 2 January 1986, see page 4, line 18 - page 5, line 6 (cited in the application -----	11

FURTHER INFORMATION C ;INUED FROM THE SECOND SHEET

V. [X] OBSERVATION WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claim numbers 20 because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1 (iv)

2. [ ] Claim numbers because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application

2. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9101332 SA 49155

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 08/11/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 9000594	25-01-90	AU-A- 3867789	05-02-90
		EP-A- 0424437	02-05-91
EP-A- 0166410	02-01-86	US-A- 4707357	17-11-87
		AU-B- 596561	10-05-90
		AU-A- 4399085	02-01-86
		JP-A- 61149093	07-07-86